Reprinted from BACTERIOLOGICAL REVIEWS
Vol. 21, No. 4, December, 1957
Printed in U.S.A.

SYMPOSIUM ON BACTERIAL SPORE GERMINATION[1]

E. STATEN WYNNE,[2] *Convener*

The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas

To our knowledge the only previous symposium of this society on the general subject of bacterial spores was that presented at the Fifty-first General Meeting at Chicago in 1951. However, of the 9 papers comprising the 1951 symposium, only a portion of 1 (presented by the convener of the present symposium) concerned spore germination. This symposium is the society's first on bacterial spore germination and reflects a greatly increased interest in the field in the past few years.

H. O. Halvorson presented evidence that germination requirements of aerobic spores may be affected by aging and/or heat shock. For rapid germination, fresh spores of *Bacillus cereus* var. *terminalis* normally required mild heat shock (15 min at 65 C), L-alanine, and adenosine. However, spores aged 6 months or more (as lyophilized cells at room temperature or wet frozen cells) often germinated in L-alanine after heat shock, in adenosine after heat shock, or without heat shock in the presence of both compounds. When fresh spores were heated at 65 C for 8 hr or more in the presence of minute concentrations of L-alanine, they resembled aged spores in being capable of germination in either L-alanine or adenosine. In fact, germination of such spores could be brought about with glucose or intermediates of the citric acid cycle. Spores subjected to heat shock in water for 4 hr germinated in adenosine, but not in L-alanine. Chromatographic analysis of water extracts of aged spores revealed the presence of L-alanine, and it was suggested that alteration of germination requirements on aging may be due to slow liberation of L-alanine from the spores. A moisture concentration higher than that for vegetative growth was required, for germination of fresh spores; this requirement was lost during storage for 1½ years in the wet frozen state.

Changes occurring during aging, and to some extent heat shock, resulted in spores becoming more like vegetative cells; these changes may in fact be stages in a complex germination process. Though L-alanine is liberated during aging, it was considered that the key substance in germination may be a compound formed from either alanine or adenosine.

B. D. Church presented findings on metabolic pathways in spores of aerobic bacilli. Reports of germination in the presence of single compounds (*e.g.*, glucose, alanine, or adenosine), together with the recent finding of an active alanine racemase enzyme in clean spores, have intensified efforts to define the initial stages of spore germination in terms of biochemical reactions. Through this approach a number of enzymatic activities have been associated with resting spores. Investigations of oxidative metabolism provide one avenue for further studies.

Clean, intact, heat-activated spores of *B. cereus* var. *terminalis* oxidized glucose, gluconate, 2-ketogluconate (2-KG), 6-phosphogluconate (6-PG), glucose-6-phosphate (G-6-P), and pyruvate. Spore extracts oxidized glucose, gluconate, 2-KG, and pyruvate. Intermediates of the Embden-Meyerhof pathway other than G-6-P and pyruvate were not oxidized, either by intact spores or spore extracts. Chromatographic identification of end products of glucose oxidation by spore extracts revealed gluconate, 2-KG, 2-keto-6-phosphogluconate (2-K-6-PG), and pyruvate. Diphosphopyridine nucleotide (DPN) was required for

[1] This symposium was held at the Fifty-seventh General Meeting of the Society of American Bacteriologists in Detroit, Michigan, on April 29, 1957. The symposium was arranged by E. Staten Wynne. Participants were: H. O. Halvorson, *University of Illinois, Urbana, Illinois;* Brooks D. Church, *Lambert-Hudnut Division of Warner-Lambert Pharmaceutical Company, Morris Plains, New Jersey;* C. F. Schmidt, *Continental Can Company, Chicago, Illinois;* E. Staten Wynne, *The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas;* and Hillel S. Levinson and Mildred T. Hyatt, *Quartermaster Research and Engineering Center, Natick, Massachusetts.*

[2] A portion of this symposium summarizes investigations of E. Staten Wynne which were supported in part by research grant C-1507 from the National Cancer Institute of the National Institutes of Health, Public Health Service.

optional glucose oxidation by spore extracts; dialyzed spore extracts required DPN, adenosine triphosphate (ATP), inorganic phosphate, and Fe^{++}.

Although 2 enzymes of the hexosemonophosphate (HMP) shunt pathway, G-6-P dehydrogenase and 6 PG dehydrogenase, were active in spore extracts, the HMP shunt was not operative because of the absence of hexokinase activity. Examination of spore extracts for gluconokinase activity was also negative. Reduction of DPN and TPN was not stimulated by phosphate. 2-KG formed from the oxidation of gluconate was phosphorylated by a typical Mg^{++}-requiring kinase. The involvement of ATP in this reaction may explain the phosphate stimulation of glucose oxidation observed in dialyzed extracts. The reactions from 2-K-6-PG (formed by phosphorylation of 2-KG) to pyruvate are not understood.

The presence in spore extracts of a system leading to pyruvate from glucose, and a second system oxidizing pyruvate, provides a basis for a supply of energy and biosynthetic intermediates. The entire system for pyruvate oxidation was associated with a particulate fraction which completely sedimented at 100,000 × G. Rapid pyruvate oxidation occurred on addition to this fraction of DPN, ATP, cocarboxylase, and $MnCl_2$ When the reaction mixture was sparked with oxalacetate (OAA), these particles also actively oxidized acetate, succinate, fumarate, and OAA. Citrate, *cis*aconitate, and malate were not oxidized. The presence of succinic dehydrogenase was indicated by competitive reversal of succinate oxidation by malonate.

It had been noted previously that the L-alanine requirement for spore germination was spared by pyruvate. Spore extracts contained a pyridoxal phosphate (B_6P)-requiring system which deaminated L- or D-alanine to NH_3 and pyruvate. When pyruvate oxidation was suppressed by 1,3-bis(ethylhexyl)-5-methyl-5-aminohexahydropyrimidine (BEP) during alanine deamination, the pyruvate level was increased. Since these extracts contained an active alanine racemase, the deamination of both L- and D-alanine may represent a nonspecificity on the part of the deaminase or a coupled reaction with the racemase.

Existence of a metabolic sequence leading to pyruvate formation, and a cycle for triose oxidation, provides a basis for the production by spores of C skeletons for amino acid synthesis during the early stages of germination—alanine from pyruvate, the aspartic acid family from OAA, and the glutamic acid family from 2-ketoglutarate. A glutamic-aspartic transaminase has been demonstrated in spores of *Bacillus megaterium*. Other amino acid synthesizing systems may be active or dormant in spores.

BEP not only inhibited pyruvate oxidation by spores, but also inhibited germination in the presence of L-alanine and adenosine. Both inhibitions were reversed by thiamine. The simplest interpretation of these results is that pyruvate or a product of pyruvate (derived from glucose, alanine, or endogenous reserves) is required for germination of spores of *B. cereus* var. *terminalis*.

C. F. Schmidt discussed activators and inhibitors of germination. In studies of these substances, results obtained depend upon the test organism, the criterion of germination, and the incubation time(s). Loss of refractility or acquisition of stainability has proved a convenient criterion of rapid germination; thermolability has been an adequate criterion when germination is slow. Since some effects are observed only upon relatively prolonged incubation, while others are apparent only with relatively short incubation, a single incubation time may fail to give a true picture.

Chemical activators reported in the literature include the amino acids L-alanine, DL-isoleucine, DL-methionine, DL-phenylalanine, DL-serine, DL-valine, and L-proline; glucose and a variety of other carbohydrates; intermediates in carbohydrate metabolism, including citrate, pyruvate, CO_2, malate, fumarate, and succinate; caramelized glucose; furfural; and the ions Cl^-, NO_3^-, and $PO_4^{\equiv}$. Inhibitors reported include rancid long chain fatty acids, arsenate, azide, dinitrophenol, fluoride, iodoacetate, malonate, and oxine. Heat activation has been described by many workers, and may at times be a necessary adjunct to the action of chemical activators. Deactivation of heated suspensions may occur in some instances, as well as reactivation on reheating.

Using Difco dextrose tryptone agar with added soluble starch, Schmidt found that only 37 per cent of the spores of a strain of *Bacillus subtilis* required heat activation with an incubation of 2 to 3 days, and 30 per cent with 5 to 10 days. On the other hand, in a synthetic medium (con-

taining asparagine, dextrose, and salts A and B), 96 per cent of the spores required heat activation with 5 to 6 days of incubation, and 84 per cent with 15 days. When heat activation was used, colony counts with the synthetic medium were equal to those obtained with the dextrose tryptone starch agar. It was therefore concluded that chemical activation of germination occurred in the complex medium. Because of the occurrence of chemical activation, studies of heat activation requirements by means of complex plating media may be unreliable.

In the synthetic counting medium, chemical activation of germination occurred promptly with L-alanine, β-alanine, and L-valine, while L-methionine and L-serine exerted effects only after prolonged incubation. D-alanine, D-valine, and D-serine inhibited activation by the corresponding L-isomers. In studies of "cross-blocking," all 3 of these D forms inhibited activation by β-alanine, and D-alanine or D-serine inhibited activation by L-valine. Since D-alanine, but not D-valine or D-serine, inhibited activation by L-alanine and by the complex dextrose tryptone starch agar, the activator in this medium may be L-alanine. The 3 D-amino acids all failed to inhibit development of heat-activated spores on the synthetic counting medium. This finding, along with the observation that some of the spores are activated by heat but not by amino acids, suggests that the mechanisms of activation by heat and by L-amino acids may be different.

E. S. Wynne presented evidence that spores of 5 otherwise mesophilic *Clostridium* species germinate at 75 C in glucose autoclaved at alkaline, or at most slightly acid, pH values. The medium used in most of the studies was prepared by autoclaving 10 per cent glucose containing 0.2 M K_2HPO_4 for 80 min at 121 C, followed by dilution of 1 to 5 or 1 to 50 and adjustment of the pH to 7.5. This medium was termed glucose autoclaved with phosphate (GAWP).

With *Clostridium botulinum* 62A and 115B, *Clostridium feseri*, *Clostridium perfringens*, and putrefactive anaerobe no. 3679, incubation at 75 C in GAWP resulted in a rapid decrease in recoverable spores as measured by colony development in a yeast extract starch bicarbonate (YESB) agar. Such a decrease conceivably might result from (a) toxicity of GAWP toward development of colonies or (b) a sporicidal effect of GAWP. However, GAWP added to YESB agar did not affect the number of colonies developing from control spores of any of the test strains. Furthermore, since GAWP added to Difco brain heart infusion broth showed no detectable toxicity toward growth of *vegetative* cells, a sporicidal effect was deemed unlikely.

The following findings were considered to suggest that the rapid decrease in recoverable spores occurring at 75 C in GAWP is the result of germination: (a) spore levels remained essentially constant during incubation for comparable periods in buffer or Difco brain heart infusion broth with BBL thioglycolate supplement; (b) in keeping with the kinetics of germination of *Clostridium* spores in a complex medium, the process in all organisms exhibited a definite lag of 2 to 15 min, after which the decrease in countable spores appeared to proceed logarithmically in 3 of 5 strains; (c) 1.0 mg per ml of sodium oleate, a known inhibitor of germination of *Clostridium* spores in complex media, significantly inhibited decrease in spore recoveries in all strains; (d) the process in all test organisms was markedly inhibited at pH 5, as is germination of these spores in a complex medium; and (e) decrease in recoverable spores was inhibited by many, but not all, of a number of metabolic analogs and cell poisons (8 of 8 purine analogs, 8 of 11 pyrimidine analogs, 2 of 2 amino acid analogs, 2 of 5 growth factor analogs, and 2 of 9 miscellaneous cell poisons).

The glucose in GAWP could be replaced by fructose, mannose, arabinose, xylose, lactose, or maltose, but not sucrose. Phosphate could be replaced by Na_2CO_3, K_2CO_3, or $CaCO_3$, but no buffer was needed as long as the initial pH was sufficiently high to insure that the pH remained above 5 during at least the major portion of the autoclaving period. It appears that autoclaving sugars at alkaline or slightly acid pH results in production of acidic substance(s) and substance(s) stimulating germination, although the two may not be identical. The nature of the substance(s) stimulating germination is unknown. Furfural and hydroxymethylfurfural supported germination at 75 C, but are known to be formed from carbohydrates chiefly at pH values of 3 or less. Glucose autoclaved at such acid pH values was inactive.

H. S. Levinson and M. T. Hyatt discussed the role of certain ions in germination and postgerminative development of *Bacillus megaterium* spores. Germination was considered to consist of

the initial changes in the transition from a spore to a vegetative cell, including loss of heat resistance, loss of refractility, increase in metabolic activity, and an increase in stainability with methylene blue. The subsequent postgerminative development may be divided into swelling, emergence, elongation, and cell division, each phase being marked by a characteristic linear rate of oxygen consumption.

Mn^{++} increased the rate and percentage of germination. Though germination occurred in buffered glucose in the absence of added Mn^{++}, the spores were found to contain 50 to 100 ppm of Mn^{++}. Mn^{++} was considered to act, at least in part, by activating spore protease or peptidase to hydrolyze spore material to amino acids or simple peptides stimulatory to germination. Proteolytic enzymes were demonstrated in spore extracts, and Mn^{++} stimulated hydrolysis by these enzymes of gelatin, egg albumin, or spore proteins. L-Alanine, a possible product of peptidases, markedly enhanced germination, and without the appreciable lag noted with Mn^{++}. Spore extracts stimulated germination, with a concentration curve similar to that of alanine, and chromatograms revealed the presence of 5 μg of alanine per 100 μg of extract solids. However, since the stimulatory action of spore extracts was roughly twice that expected from the alanine content, the role of Mn^{++} may not be confined to production of L-alanine, or even to proteolysis.

Added sulfur in the form of $SO_4^{=}$, $SO_3^{=}$, $S_2O_3^{=}$, cysteine, or methionine was essential for postgerminative development. Though the spores contained 0.3 per cent sulfur, it could not be utilized by the germinated spore. The role of added sulfur in postgerminative development has not been determined, but the low concentration (3.2 ppm) required to exert a maximum effect and the immediate further development of germinated spores on the addition of sulfur suggest its utilization in the synthesis of substances active in catalysis (enzymes, coenzymes).

Spore germination was not stimulated or inhibited by the cations K^{+}, Mg^{++}, Zn^{++}, Cu^{++}, Fe^{++}, Co^{++}, and Ni^{++}. However, Co^{++} and Ni^{++} inhibited postgerminative development. Co^{++} was inhibitory to stages of postgerminative development prior to emergence, but not to elongation or cell division; Ni^{++} was inhibitory to all stages except cell division. Such differences in sites of inhibitory effect may prove of value in separation of the metabolic requirements of each postgerminative stage of development.